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(54) Title: REGENERATION OF COTTON PLANTS

(57) Abstract: The present invention provides methods for the regeneration of a cotton plant comprising culturing a protoplast prepared from somatic cotton plant tissue in a culture medium, wherein the protoplast is embedded in a solid medium; and regenerating a plant from the cultured protoplast. The invention also provides method for the genetic transformation of a cotton plant comprising culturing a protoplast prepared from somatic cotton plant tissue in a culture medium to form callus, wherein the protoplast is embedded in a solid medium; introducing foreign DNA into the callus to form transformed callus; and regenerating a plant from the transformed callus. The invention further provides plants produced according to the methods of the invention, and seeds and progeny of such plants.

REGENERATION OF COTTON PLANTS

FIELD OF THE INVENTION

The present invention relates to methods of regenerating cotton plants from transformed or nontransformed cotton protoplasts.

BACKGROUND OF THE INVENTION

Although the propagation of many agronomically important crop plants *in vitro* through tissue culture is now commonplace, the routine culture and regeneration of cotton plants remains difficult.

The most widely used system for whole plant regeneration of cotton by somatic embryogenesis was first developed by Trolinder et al., Plant Cell Reports 6: 231-234, 1987. Unfortunately, while a wide range of cultivars were screened, only a few related varieties of cotton were amenable to this type of regeneration methodology. Moreover, none of these cultivars have agonomic significance. Therefore, any genetic engineering projects employing this embryogenesis strategy with a Coker or other responsive line must incorporate an extensive lengthy conventional breeding program to transfer added-value gene traits into agronomically useful germplasm.

Several groups have reported regeneration of cotton plants from protoplasts isolated from embryogenic suspension cell cultures. Peeters, M.C. and Owennen, R. "Regeneration of Plants from Cotton Protoplasts", Biotechnology in Agriculture and Forestry, 42: 48-59, 1998, discloses a method for regenerating cotton plants using protoplasts isolated from embryogenic cell suspension cultures wherein the protoplasts are cultured on a feeder layer containing a Coker embryogenic cell suspension. Zhixian et al. Current Plant Science and Biotech in Agriculture 15: 283-286, 1993 and She et al., Biotechnology in Agriculture and Forestry 34: 63-69, 1995 also disclose cotton plant regeneration from protoplasts isolated from embryogenic suspension cell cultures.

Other methods of regenerating cotton plants start with either pre-existing meristematic tissue or transition region tissue. WO 98/15622 discloses transformation and regeneration of fertile cotton plants, starting with transition region tissue, i.e. tissue from the region where the vascular system of the root and shoot change organization. WO 97/43430 discloses a method of regenerating cotton plants using explants of apical and/or nodal meristematic tissues wherein shootlets are induced to proliferate from the explant by culturing the explant with nutrient media supplemented with cytokinin followed by rooting the shootlet.

Despite some success, rapid, reliable regeneration of cotton plants remains difficult. Present methods of cotton plant regeneration have drawbacks that limit their usefulness. Some can be used successfully only with a few varieties of cotton that are not agriculturally important. Methods starting with pre-existing meristematic tissue often have a high incidence of chimeras and the transformation frequency is low. In view of the drawbacks and difficulties of present methods, there exists a need for novel methods of regenerating cotton plants.

SUMMARY OF THE INVENTION

The present invention provides methods for the regeneration of a cotton plant comprising culturing a protoplast prepared from somatic cotton plant tissue in a culture medium, wherein the protoplast is embedded in a solid medium; and regenerating a plant from the cultured protoplast.

Another aspect of the invention provides methods for the production of a transgenic cotton plant comprising introducing foreign DNA into a protoplast prepared from somatic cotton plant tissue, culturing the protoplast in a culture medium, wherein the protoplast is embedded in a solid medium; and regenerating a transgenic plant from the cultured protoplast.

A further aspect of the invention provides methods for the production of a transgenic cotton plant comprising culturing a protoplast prepared from somatic cotton plant tissue in a culture medium to form callus, wherein the protoplast is embedded a solid medium; introducing foreign DNA into the callus to form transformed callus and regenerating a transgenic plant from the transformed callus.

The invention also provides cotton plants produced according to the methods of the invention, and seeds and progeny of such plants.

These and other aspects of the invention are more fully described in the following detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel methods of regenerating cotton plants from protoplasts, cotton plants produced according to the methods of the invention disclosed herein, and seeds and progeny of such plants.

In one aspect, the invention provides a method of producing a cotton plant comprising culturing a protoplast prepared from somatic cotton plant tissue in a culture medium, wherein the protoplast is embedded in a solid medium; and regenerating a plant from the cultured protoplast. Culturing the protoplasts embedded in a solid medium, preferably in the presence of an antioxidant, produces microcalli which are then regenerated into plants. Optionally, the protoplast may be transformed to contain foreign DNA, so that the method can be used to produce genetically modified or transformed plants. Alternatively, in a further aspect of the invention, callus produced from the protoplast can be transformed with foreign DNA prior to regeneration into a plant.

Protoplasts offer several advantages as a starting material for regeneration of plants. Protoplasts are amenable to direct DNA transfer methods such as polyethylene glycol (PEG), electroporation or microinjection, and transformed protoplasts offer the prospect of producing nonchimeric plants as they regenerate from a single cell. When plants are regenerated directly from pre-existing meristematic tissue the incidence of chimera formation is high. In addition, the transformation frequency of pre-existing meristematic tissue is low, resulting in lower rates of transformed plants. The single cell origin of plants regenerated from protoplasts is also useful in the development of mutants with valuable characteristics resulting from somaclonal variation or from induced mutagenesis during tissue culture.

The methods of the invention can be performed with any species of *Gossypium*, including the agriculturally important species *G. hirsutum* and *G. barbadense*.

Protoplasts used in the methods herein are isolated from somatic cotton plant tissues. Somatic cotton tissues refers to differentiated cotton plant structures including (but not restricted to) cotyledon, leaf, stem or roots. Prior methods of regenerating cotton plants from protoplasts have been restricted to using proliferating callus and suspension cell cultures, previously selected for their predisposition to be embryogenic, as the starting material. In contrast, the plant tissues used in the Applicants' methods constitute fully differentiated plant organs removed directly from a developing plant. Preferably, the tissue used in the methods of the invention is cotyledon tissue, more preferably the tissue is explants taken from four to five day old cotyledons, when the cotyledons are at the stage where they are green but still partly folded (i.e., not fully expanded to a flat plateau shape) is preferable. Preferably, the petioles and major cotyledonary veins are removed before the tissue is used for protoplast isolation.

Protoplasts are then prepared from the explant of somatic cotton tissue. Preferably, protoplasts are isolated from the explant through enzymatic digestion of the cell wall, but any isolation method that provides viable protoplasts can be used. In a preferred embodiment of the invention, a digestion medium comprised of CPW salts (Frearson, E.M. et al., Dev. biol. 33: 130-137, 1973), mannitol, n-propylgallate (n-PG) and the semi-purified cell wall digesting enzyme preparations Cellulase R10, Macerozyme R-10 (Yakult-Honsha, Tokyo, Japan) and Driselase, (Sigma Chemical Company, St Louis Missouri, USA) is used to digest the cell wall and protoplasts are then isolated by centrifugation. Prior to isolation of protoplasts, the explant tissue can be cultured in a preincubation medium comprised of CPW salts supplemented with mannitol, calcium chloride and n-propylgallate. Viability of the protoplasts can be determined using a standard fluorescein diacetate (FDA) staining test (Widholm, J.M., Stain Technology 47: 189-194, 1972).

The isolated protoplasts are then embedded in a solid medium, preferably alginate, at low density (approximately 25,000 to 50,000 protoplasts in 1ml

alginate), and cultured in a culture medium. The number of protoplasts per milliliter of alginate can be altered to provide optimal rates of callus formation. In a preferred embodiment of the invention, protoplasts are mixed with sodium alginate and the mixture is solidified over calcium agar. The gelled, solidified calcium alginate is removed from the agar and the resultant protoplasts embedded in alginate are cultured in culture medium containing an antioxidant.

The culture medium for the embedded protoplasts is preferably K8p medium as described in Kao and Michayluk, Planta 126: 105-110, 1975, lacking Sequestrene and casamino acids, and supplemented with an antioxidant, ethylene diaminetetraacetic acid (EDTA), ferrous sulfate and glucose (modified K8p medium). Preferably, the antioxidant is n-propyl gallate or glutathione, more preferably n-propyl gallate. However, it may be possible to culture the cells in the presence of other antioxidants such as vitamin E, superoxide dismutase or catalase. The culture medium also preferably contains one or more phytohormones, such as naphthalene acetic acid (NAA) and zeatin. The pH of the medium is between about 5.5 and 6.0, preferably 5.8. A preferred protoplast culture medium contains between about 0.1 mg/L to about 2mg/L NAA and 0.1 mg/L to about 2.4 mg/L zeatin. With some varieties of cotton, it may be desirable to use much lower concentrations of phytohormones to obtain good yields of protoplast-derived colonies.

The embedded protoplasts are cultured in the dark at a temperature of from about 28 to 32°C. Applicants have found that culturing the protoplasts at 32°C provides plating frequencies of approximately 50%. For some cotton varieties, however, larger numbers of protoplast-derived colonies or microcalli may be produced when the protoplasts are cultured at 30°C. After 2-3 days, the embedded protoplasts divide rapidly and proceed initially at a rapid rate to form microcolonies of cells which are visible to the naked eye after about fourteen days. The embedded protoplasts are maintained in the protoplast culture medium for about fourteen to thirty-five days until protoplast derived colonies or microcalli begin to break out of the solid medium in which the protoplasts are embedded. The protoplast-derived colonies or microcalli are then transferred to solid callus culture medium. Transfer of the protoplast-derived colonies is accomplished by cutting the solid medium

containing the colonies into small pieces and placing them on a solid callus culture medium.

A preferred callus culture medium is comprised of solidified MS medium (Murashige and Skoog, Physiol. Plant. 15: 473-497, 1962) supplemented with glucose, an auxin and a cytokinin. Suitable auxins include indoleacetic acid (IAA) and naphthylene acetic acid (NAA). The amount used to supplement the MS medium will depend on the auxin used. 0.5-2.0 mg/L IAA and 1 mg/L NAA have been found to be effective. Suitable cytokinins include kinetin. The amount of cytokinin used to supplement the MS medium will depend of the cytokinin used. 0.1 mg/L to about 0.5 mg/L kinetin has been found to be effective. The medium can be supplemented with 1-5% glucose, preferably 3% glucose. The pH of the medium is between about 5.5 to 6.0, preferably 5.8. The supplemented MS medium is solidified with a gelling agent such as Phytagel (Sigma Company, St. Louis, Missouri, USA).

Cultures are maintained in darkness at a temperature between about 28°C - 32°C, preferably at 30°C, until individual colonies reach a size of 1-3 millimeters. At this time the colonies can be picked off and transferred to fresh callus culture medium. The calli are then cultured under the same conditions until signs of somatic embryogenesis are observed. The calli are preferably subcultured every four weeks during this time. Cultures can be maintained in darkness or in low intensity (2000 lux) light.

Callus morphology changes with the age of the culture, usually beginning white and watery and ending up light green to brown and crumbly/hard.

Somatic embryogenic callus may be observed, microscopically or by visual inspection, after about twenty weeks of culture in callus culture medium. The somatic embryogenic callus has a distinct globular appearance and is usually white or pale green in color, often with areas of (intense) red pigmentation. The areas of somatic embryogenic callus are then removed and either placed on the medium next to the source callus, or in a separate dish.

Somatic embryogenic calli are maintained on a solidified MS medium (Murashige and Skoog, Physiol. Plant 15: 473-497, 1962), supplemented with

potassium nitrate and glucose, which is solidified with a gelling agent, such as Phytagel. The medium has a pH between about 5.5 to 6.0, preferably 5.8. The calli are preferably subcultured every four weeks by spreading thinly over the surface or fresh plates.

Embryos form spontaneously from the embryogenic callus and when developing calli reach a size of approximately three millimeters, they are transferred to desiccation medium. The desiccation medium is preferably comprised of Steward medium supplemented with sucrose and agar. The desiccation medium has a pH of about 6.5 to 7.0, preferably 6.8.

After about fourteen days in darkness, the developing embryos are transferred to pots containing development medium and cultured in the light until an extensive root system and the first three true leaves have formed. Plant development medium is preferably comprised of Stewards medium supplemented with Phytagel, agar and sucrose. The pH of the development medium is from about 6.5 to 7.0, preferably 6.8.

On appearance of the third true leaf, plantlets with two roots at least 1cm long are transferred to pots containing potting medium, typically compost, and grown initially under high humidity and then under normal greenhouse conditions.

Transformed or genetically modified plants can also be prepared using the methods of the invention. Introduction of foreign DNA can be accomplished by transforming the protoplasts, or by transforming the callus formed from the protoplasts. The callus itself can be used for transformation, or a suspension cell culture can be prepared from the callus and used for transformation. Suspension cell cultures can be readily prepared from the callus by culturing the callus in a liquid medium such as the callus culture medium disclosed herein lacking the gelling medium.

Thus, another aspect of the invention provides methods for producing transgenic or genetically modified plants comprising the steps of introducing foreign DNA into a protoplast prepared from somatic cotton plant tissue, culturing the protoplast in a culture medium, wherein the protoplast is embedded in a solid medium; and regenerating a transgenic plant from the cultured protoplast. A further

aspect of the invention provides methods of preparing transgenic or genetically modified plants comprising the steps of culturing a protoplast prepared from somatic cotton plant tissue in a culture medium to form callus, wherein the protoplast is embedded in a solid medium; introducing foreign DNA into the callus to form transformed callus; and regenerating a plant from the transformed callus.

As discussed above, plants regenerated from protoplasts have the advantage of producing non-chimeric plants as they regenerate from a single cell. Additionally, protoplasts are amenable to methods of transformation such as PEG, electroporation and microinjection that are not usually possible when cells with walls are used for regeneration of whole plants. For some applications of the present invention, however, it may be preferable or advantageous to transform the calli produced from the protoplasts, or to transform the protoplasts twice, first as protoplasts and later after production of calli from the protoplasts.

The methods of the invention are generally applicable to the preparation of transgenic plants expressing any type of foreign gene. The description of foreign genes, selection methods and transformation methods set out hereinbelow are exemplary only and are not intended to limit the type or number of foreign genes that can be incorporated into a plant using the methods of the invention. Protoplasts and callus can be transformed with foreign DNA by conventional methods. Selection of cells or plants expressing a particular foreign gene can be accomplished by methods known in the art relating to such gene and its product.

As used herein, foreign DNA refers to DNA originally isolated from an organism other than cotton. Foreign DNA can also be DNA originating in cotton and reintroduced into cotton by transformation or other recombinant methods, or DNA prepared by chemical synthesis. Generally, the foreign DNA will be a gene, but can also contain other elements such as promoters, and regulatory elements. A foreign gene is thus any gene that has been isolated from an organism other than cotton, or originally isolated from cotton and reintroduced into cotton by transformation or other recombinant method, or prepared synthetically.

The form of the foreign DNA when inserted into the protoplasts or callus will depend on the requirements of the transformation method. For most methods,

the foreign DNA will comprise at least one gene and will be incorporated into an expression vector. Such an expression vector can be a previously made construct or it can be constructed from known DNA sequences by genetic engineering methods well known to one skilled in the art. The nucleotide sequence of the foreign gene can be optimized for expression in plants by modifying the codon usage to include plant preferred codons. See, for example, Murray et al., NAR 17: 477 (1989). The expression vector can contain a gene that can serve as a selectable or screenable marker for transformation, a gene sequence that confers a desired trait to a transgenic plant, or any combination of such genes. It should be understood, however, that foregoing categories are for convenience of discussion and not exclusive of the use of a particular gene, and that a gene may be used as both a selectable marker and for conferring a trait of interest to the plant.

Additionally, the expression vectors will contain promoters and regulatory elements. The foreign genes are operably linked to promoters active in cotton such as the CaMV 35S. nopaline or octopine synthase promoters. See Vontling et al., Mol. Plant-Microbe Interactions 4(4): 370-378 (1991). Any other plant promoter active in cotton can be used including inducible, tissue-specific, tissue-preferred or constitutive promoters such as the promoters described above. The expression vector can also contain regulatory sequences such as transcription termination sequences, polyadenylation sequences, and possibly exons. Selection of the type of regulatory elements and arrangement in the expression vector well known to one skilled in the art. Depending on the desired function of the gene, secretion or cellular compartmentalization sequences are added. The methods of DNA manipulation involved in preparing the expression vectors are standard, and well known to one skilled in the art.

Numerous methods for plant transformation have been developed. See, for example, Miki et al., "Procedures for Introducing Foreign DNA into Plants" in Methods in Plant Molecular Biology and Biotechnology, Glick, B.R. and Thompson, J.E. Eds. (CRC Press, Inc., Boca Raton, 1993) pages 67-68.

A widely utilized method for introducing an expression vector into plants is based on the natural transformation system of *Agrobacterium*. See, for example,

Horsch et al., Science 227:1229 (1985). *A. tumefaciens* and *A. rhizogenes* are plant pathogenic soil bacteria which genetically transform plant cells. The Ti and Ri plasmids of A. tumefaciens and A. rhizogenes, respectively, carry genes responsible for genetic transformation of the plant. The Ti plasmid has the natural ability to transfer a segment of itself, referred to as the transfer DNA (T-DNA) region, into the genome of infected plant cells. *Agrobacterium* vector systems and methods for Agrobacterium-mediated gene transfer well known and are provided, for example, in Gruber et al. "Vectors for Plant Transformation" in *Methods in Plant Molecular Biology and Biotechnology*, Glick, B.R. and Thomson, J.S. Eds. (CRC Press, Inc., Boca Raton, 1993) pages 80-119, and Moloney et al. Plant Cell Reports 8: 238 (1989).

Several methods of plant transformation, collectively referred to as direct gene transfer, can also be used, particularly with protoplasts.

A generally applicable method of plant transformation is microprojectile-mediated transformation wherein DNA is carried on the surface of microprojectiles measuring 1 to 4 μm. The expression vector is introduced into plant tissues with a biolistic device that accelerates the microprojectiles to speeds of 300 to 600 m/s which is sufficient to penetrate plant cell walls and membranes. See, for example, U.S. patents 5,478,744; U.S. patent 5,371,015; and U.S. patent 4,945,050.

Yet another technology for production of transgenic plants is whisker-mediated transformation whereby certain materials, when incubated with plant tissue, facilitates entry of DNA molecules into plant cells. Wang et al., In Vitro Cell. Dev. Biol. 34: 101-4 (1995).

Another method for physical delivery of DNA to plants is sonication of target cells. Zhang et al., Bio/Technology 9: 996 (1991). Alternatively, liposome or spheroplast fusion have been used to introduce expression vectors into plants. Deshayes et al., EMBO J., 4: 2731 (1985), Christou et al., Proc Natl. Acad. Sci. U.S.A. 84: 3962 (1987). Direct uptake of DNA into protoplasts using $CaCl_2$ precipitation, polyvinyl alcohol or poly-L-ornithine have also been reported. Hain et al., Mol. Gen. Genet. 199: 161 (1985) and Draper et al., Plant Cell Physiol. 23: 451 (1982). Electroporation of protoplasts and whole cells and tissues have also

been described. Donn et al., In Abstracts of VIIth International Congress on Plant Cell and Tissue Culture IAPTC, A2-38, p 53 (1990); D'Halluin et al., Plant Cell 4: 1495-1505 (1992) and Spencer et al., Plant Mol. Biol. 24: 51-61 (1994).

Expression vectors may include at least one genetic marker that allows transformed cells to be either recovered by negative selection, i.e., inhibiting growth of cells that do not contain the selectable marker gene, or by screening for product encoded by the genetic marker. Many of the commonly used selectable marker genes for plant transformation were isolated from bacteria and code for enzymes that metabolically detoxify a selective chemical agent which may be an antibiotic or a herbicide. Other selectable marker genes encode an altered target which is insensitive to the inhibitor.

A commonly used selectable marker gene for plant transformation is the neomycin phosphotransferase II (nptII) gene which confers resistance to kanamycin. Fraley et al., Proc. Natl. Acad. Sci. U.S.A., 80: 4803 (1983). Another commonly used selectable marker gene is the hygromycin phosphotransferase gene which confers resistance to the antibiotic hygromycin. Vanden Elzen et al., Plant Mol. Biol., 5: 299 (1985).

Additional selectable marker genes of bacterial origin that confer resistance to antibiotics include gentamycin acetyl transferase, streptomycin phosphotransferase, aminoglycoside-3'-adenyl transferase, and the bleomycin resistance determinant. Other selectable marker genes confer resistance to herbicides such as glyphosate, glufosinate, broxymil, or tabtoxine ß-lactam.

Other selectable marker genes that are not of bacterial origin are also available. These genes include, for example, mouse dihydrofolate reductase, plant 5-enolpyruvylshikimate-3-phosphate synthase and plant acetolactate synthase.

Another type of marker gene for plant transformation requires screening of presumptively transformed plant cells rather than direct genetic selection of transformed cells for resistance to a toxic substance such as an antibiotic. Commonly used genes for screening presumptively transformed cells include beta-glucuronidase (GUS), beta-galactosidase, luciferase and chloramphenicol acetytransferase.

The methods of the invention can be used to introduce any type of desired trait into cotton plants. For example, the foreign gene may confer resistance to pests or disease or plants that are resistant to specific pathogen strains. See, for example Geiser et al., Gene 48: 109 (1986) (a Bacillus thuringiensis protein, a derivative thereof or a synthetic polypeptide modeled thereon). For a review of Bt δ-endotoxin genes see Kelly, et al., Pesticide-Producing Bacteria, in Molec. Biol. and Biotech., ed. Meyers, VCH Publishers, New-York, pages 668-72 (1995).

The foreign gene can be an enzyme inhibitor, for example, a protease or an amylase inhibitor. See, for example, Abe et al., J. Biol. Chem. 262: 16793 (1987) (nucleotide sequence of rice cysteine proteinase inhibitor), Huub et al., Plant Molec. Biol. 21: 985 (1993) (nucleotide sequence of cDNA encoding tobacco proteinase inhibitor I), and Sumitani et al., Biosci. Biotech. Biochem. 57: 1243 (1993) (nucleotide sequence of Streptomyces nitrosporeus α-amylase inhibitor). The foreign gene can also be an insect-specified hormone or pheromone such as an ecdysteroid or juvenile hormone, or an antagonist or agonist thereof; an insect-specified peptide or neuropeptide which, upon expression, disrupts the physiology of the affected pest; or an insect-specific venom produced in nature by a snake, a wasp, etc. The foreign gene can provide insecticidal activity by producing a hyperaccumulation of a monterpene, a sesquiterpene, a steroid, hydroxamic acid, a phenylpropanoid derivative or another non-protein molecule with insecticidal activity; or provide insecticidal activity by expressing an enzyme involved in the modification, including the post-translational modification, of a biologically active molecule; for example, a glycolytic enzyme, a proteolytic enzyme, a lipolytic enzyme, a nuclease, a cyclase, a transaminase, an esterase, a hydrolase, a phosphatase, a kinase, a phosphorylase, a polymerase, an elastase, a chitinase and a glucanase, whether natural or synthetic.

Additional examples of foreign genes include genes encoding a viral-invasive protein or a complex toxin derived therefrom. For example, the accumulation of viral coat proteins in transformed plant cells imparts resistance to viral infection and/or disease development effected by the virus from which the coat protein gene is derived, as well as by related viruses. Coat protein-mediated

resistance has been conferred upon transformed plants against alfalfa mosaic virus, cucumber mosaic virus, tobacco streak virus, potato virus X, potato virus Y, tobacco etch virus, tobacco rattle virus and tobacco mosaic virus.

It may be desirable to express the foreign gene in a plant to confer resistance to a herbicide such as glyphosate (resistance imparted by mutant EPSP synthase) and other phosphono compounds such as glufosinate (pat and bar genes), and pyridinoxy or phenoxy propionic acids and cyclohexones (inhibitor-encoding genes). Exemplary of genes conferring resistance to phenoxy propionic acids and cyclohexones, such as sethoxydim and haloxyfop, are the Acc1-S1, Acc1-S2 and Acc1-S3 genes.

The foreign gene may encode a herbicide that inhibits photosynthesis, such as a triazine (psbA and gs+ genes) and a benzonitrile (nitrilase gene).

In addition to the foregoing types of foreign genes, other types of foreign genes that may be used to prepare transgenic plants in accordance with the methods of the invention include foreign genes that may enhance the value of the plant. For example, the foreign gene may be an antisense gene that modifies fatty acid metabolism. The foreign gene may encode a protein that modifies the properties of cotton fiber; or can be used to engineer cotton lines that are male sterile thereby facilitating construction of cotton hybrids; or encode a product which disrupts pollen development.

The expression vectors can contain a constituitive or inducible promoter. Many different constitutive promoters are known in the art. Exemplary constitutive promoters include the promoters from plant viruses such as the 35S promoter from CaMV, the promoters from such genes as rice actin, or ubiquitin.

Exemplary inducible promoters include that from the ACE1 system which responds to copper; the Tet repressor from Tn10 and the *alc* promoter

Tissue-specific or tissue-preferred promoters can be utilized in the expression vector. Exemplary tissue-specific or tissue-preferred promoters include a root-preferred promoter such as that from the phaseolin gene; a leaf-specific and light-induced promoter such as that from *cab* or *rubisco*; or an anther-specific promoter such as that from LAT52.

A further aspect of the present invention provides plants produced according to the methods of the invention. The invention also provides seed from plants produced according to the methods of the invention and the progeny of plants produced according to the methods of the invention.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLE 1

1.1 STERILIZATION OF THE STARTING MATERIAL

Commercial cotton seed of the variety Coker 312 (*G. hirsutum*) was pretreated by washing for 10 min in a large volume (e.g. 400 ml / 100 seeds) of tap water with vigorous stirring to remove the fungicidal coating. The seed was then transferred to a tea strainer and was submerged in 70% ethanol for 60 sec with regular agitation. The seed was further sterilised by submerging for 25 min in a solution of sodium hypochlorite diluted with distilled water to give a final concentration of 2% free chlorine. One drop of Tween 20 was added per 100 ml to aid the wetting process. The seed was continuously agitated to maximize contact with the sterilant. After sterilization the seed was thoroughly washed in 3 x 400 ml sterile distilled water for 5, 10 and finally 15 min after which it was transferred to a sterile Petri dish for further use.

1.2 SEED GERMINATION

Glass culture jars (total volume 400 ml, 9 cm tall) containing 40 ml germination medium were used. The germination medium consisted of Stewards medium (Steward & Hsu, 1977, Planta 137, 113-117) without sucrose and solidified using 2 g / 1 Phytagel (Sigma, St. Louis, USA). Eight seeds per pot were placed on top of the medium and were cultured at 25°C in the light (2500 lux, Philips TLD 84° fluorescent) for 4 - 5 days. To assist the germination process, once 1 cm of radicle had emerged from a seed this was pushed into the germination medium to aid seedling establishment. After 4 d (or 5 d, depending on the interexperimental

variation in the speed of germination) the cotyledons had fully emerged but, were still approximately half way through the expansion phase, were still partially folded and were not yet dark green. At this stage the cotyledons were harvested for protoplast isolation.

1.3 PROTOPLAST ISOLATION

All solutions for the isolation and culture of cotton protoplasts were filter sterilised before use. Only cotyledons at the required specific developmental stage were selected as the starting material for protoplast isolation. These were harvested and the petioles and the 4-6 major cotyledonary veins / cotyledon were removed. The remaining lamina tissue was transferred to a 9 cm Petri dish (Greiner, Tissue Culture Quality) containing 10 ml preincubation medium. Approximately 12 cotyledons (6 seedlings) were needed to obtain the required 1 g tissue for each isolation. The preincubation medium comprised CPW salts (Frearson EM, Power JB & Cocking EC 1973, Dev Biol 33, 130-137) supplemented with 9% mannitol, 3.8% $CaCl_2 2H_2O$ and 0.1 mM n-propyl gallate (nPG), pH 5.8. Using a new scalpel blade the cotyledon material was finely chopped into 1-2 mm pieces and maintained at room temperature for 30 - 60 min. The preincubation medium, now also containing the broken cell debris, was then removed and replaced with the digestion solution. This consisted of CPW salts, 9% mannitol, 5mM 2-(N-morpholino) ethane sulfonic acid (MES), 0.1 mM nPG and the cell wall digesting enzymes Cellulase R-10 (0.5%, Yakult-Honsha, Tokyo, Japan) and Macerozyme R-10 (0.1%, Yakult-Honsha, Tokyo, Japan) and Driselase (0.2%, Sigma, St. Louis USA). All solutions were adjusted to pH 5.8 and were filter sterilized before use. Protoplast release occurred overnight at 25°C in the dark on a slow rotary shaker (35 rpm, 1.5 cm amplitude). Before purification the suspension was induced to disintegrate further by sucking up and down in a wide-mouthed pipette and the suspension was filtered through 250 and 55 µm nylon filters. The viable protoplasts were purified first by centrifugation at 55 x g for 5 min. The pellet was then resuspended in 10 ml CPW containing 9% mannitol and 0.1 mM nPG and centrifuged again. The second pellet was resuspended in CPW containing 15% sucrose and 0.1 mM nPG on top of

which was carefully layered in 1 ml aliquot of 9% mannitol supplemented with 1 mM calcium chloride. After a third centrifugation the viable protoplasts could be collected in the upper layer of liquid. Protoplast yield was determined using a haemocytometer and when necessary, the viability was determined using a standard FDA staining test (Widholm JM, Stain Technol. 47, 189-194, 1972).

1.4 PROTOPLAST CULTURE

Before culture, protoplasts were embedded in calcium alginate. Firstly, the protoplast density was adjusted to 100,000 protoplasts / ml mannitol solution and was carefully but very thoroughly mixed 1:1 with a filter sterilized solution of 2% sodium alginate (Sigma, St. Louis, USA). Aliquots (1 ml) were then poured onto a Ca agar (0.9% Daichin agar, 7.25% mannitol and 50 mM $CaCl_2 2H_2O$; 5 ml per 6 cm Petri dish) and left to gel for 1 h. The Ca alginate discs (now 1% Ca alginate each containing 50,000 protoplasts) were then removed and transferred to 6 cm Petri dishes (Greiner, TC quality) containing 4 ml culture medium. Dishes were sealed with a double layer of Parafilm and cultured in the dark at 32°C (*G. hirsutum*) or 30°C (*G. barbadense*). The culture medium comprised modified K8p medium as described by Kao & Michayluk 1975 (Planta 126, 105-110) but lacking Sequestrene and Casamino acids and supplemented with 0.1 mM nPG, 37 mg/l Na_2EDTA, 28 mg/l $FeSO_4 7H_2O$ and 6.84% glucose, pH 5.8. The phytohormone supplement comprised NAA (2 mg/l) and Zeatin (0.8mg/l). After 14 days the cultures could be transferred to solid medium.

1.5 CALLUS CULTURE

When the protoplast derived colonies began to break out of the alginate the discs were cut into 5 mm broad strips and these were transferred to callus culture medium. This medium comprised MS medium (Murashige & Skoog, 1962, Physiol Plant. 15, 473-497) supplemented with 3% glucose (filter sterilized and added after autoclaving), an auxin - 0.5 - 2.0 mg/l IAA or 1 mg/l NAA and a cytokinin - 0.1 mg/l kinetin, pH 5.8. The medium was solidified with 2 g/l Phytagel. Cultures were maintained at 30°C in darkness until the individual colonies had reached a size of 1 -

3 mm. At this time these were picked off and transferred to fresh medium (100 colonies / 9 cm Petri dish / 20 ml medium). Calli were subcultured (20 / dish) after 4 weeks and were subsequently maintained under these conditions with 4-weekly subcultures until signs of somatic embryogenesis were observed.

1.6 PLANT REGENERATION

After approximately 20 weeks the first signs of somatic embryogenic callus were observed either microscopically or with the naked eye. This callus had a distinct globular appearance and was usually white or pale green in colour, often with areas of (intense) red pigmentation. These areas were immediately removed on identification and were either placed on the medium next to the source callus or, when sufficient was available, on a new dish together with other pieces. These somatic embryogenic calli were maintained on Murashige & Skoog medium supplemented with 1.9 g/l KNO_3 30 g/l glucose, 2 g/l Phytagel, pH 5.8 and were subcultured every 4 weeks by spreading thinly over the surface of fresh plates. Spontaneous embryo formation was regularly observed and developing embryos were transferred to desiccation medium when they reached a size of approximately 3 mm. Desiccation medium comprised Stewards medium supplemented with 2% sucrose and 2% agar (Daichin, Tokyo, Japan), pH 6.8. After 14 d in darkness these embryos were transferred to 200 ml pots containing 40 ml plant development medium and cultured in the light until an extensive root system and the first 2 - 3 true leaves had formed. Plant development medium comprised Stewards medium supplemented with 0.15% Phytagel, 0.5% Agar and 0.5% sucrose, pH 6.8.

1.7 TRANSFER TO SOIL

On appearance of the third true leaf, plantlets with two roots at least 1 cm long were removed from the culture medium, rinsed under tap water to remove adhering agar and were transferred to moist potting compost. After a period of 7 d at high humidity the plants were weaned off without any difficulty by gradually reducing the humidity over a period of 7 d. All plants survived and were grown

further under normal greenhouse conditions (23°C day / 20°C night, 16 hour day, sunlight plus supplementary lighting when needed).

EXAMPLE 2 - EFFECT OF HORMONE BALANCE AND TEMPERATURE ON PROTOPLAST DEVELOPMENT

2.1 Coker 312 (G. hirsutum)

Protoplasts were prepared from *Gossypium hirsutum* cotyledons following the method of Example 1, sections 1.1 - 1.3. The protoplasts were then cultured by the method of Example 1, section 1.4 except that the protoplasts were cultured with varying concentrations of NAA (0.1, 0.5, 1 or 2 mg/L) and kinetin (0.1, 0.5, 1 or 2 mg/L). Very good yields of protoplast-derived colonies or microcalli were obtained with 1 mg/L NAA and 0.1 mg/L, 0.5 mg/L, 1.0 mg/L or 2 mg/L kinetin. Good yields of protoplast-derived colonies were obtained with 0.5 mg/L NAA and 0.5 mg/L, 1 mg/L or 2 mg/L kinetin. The microcolonies obtained were further cultured in accordance with the method of Example 1 and plants were obtained.

2.2 *Gossypium barbadense*

Protoplasts were prepared from *G. barbadense* cotyledons following the method of Example 1, sections 1.1 - 1.3. The protoplasts were then cultured by the method of Example 1, section 1.4 except that the protoplasts were divided into two groups and cultured at 30°C or 32° with varying concentrations of NAA (0.1, 0.5, 1 and 2 mg/L) and zeatin (0.4, 0.8, 1.2 and 2.4 mg/L). Culturing *G. barbadense* protoplasts at 30°C with 0.1 mg/L NAA and either 0.4 or 0.8 mg/L zeatin produced the greatest number of protoplast derived microcolonies. Culturing at 30°C with 0.5 mg/L NAA and 0.4, 0.8, or 1.2 mg/L zeatin or 0.1 mg/L NAA with 1.2 or 2.4 mg/L zeatin also produced acceptable numbers of protoplast derived microcolonies. The yield of protoplast microcolonies per plate was approximately 3 x 10^6/g fresh weight compared with 2.5 - 2.7 x 10^6/ g fresh weight for Coker 312 protoplasts. The microcolonies died by 7-10 days after plating. A lower plating density for *G. barbadense* is contemplated.

The experiment was repeated with lower concentrations of NAA (0.01, 0.05 and 0.1 mg/L) and zeatin (0.1, 0.2, 0.4 and 0.6 mg/L). Culturing the protoplasts at 30°C with 0.01 mg/L NAA and 0.1, 0.2, 0.4 or 0.6 mg/L zeatin produced very good

yields of protoplast-derived microcolonies. Culturing the protoplasts at 30°C with 0.05 mg/L NAA and 0.2, 0.4 or 0.6 mg/L zeatin produced good yields of protoplast colonies. Most of the protoplast-derived colonies survived and were transferred to solid callus culture medium in accordance with the method of Example 1.

2.3 Siokra S324 (*G. hirsutum*)

Protoplasts were prepared from Siokra S324 cotyledons following the method of Example 1, sections 1.1 - 1.3. The protoplasts were then cultured by the method of Example 1, section 1.4 except that the protoplasts were divided into two groups and cultured at 30°C or 32°C with varying concentrations of NAA (0.1, 0.5, 1 and 2 mg/L) and zeatin (0.4, 0.8, 1.2 and 2.4 mg/L). Greatest numbers of protoplast derived colonies were produced when the protoplasts were cultured at 32°C with 1 mg/L NAA and 0.4 mg/L zeatin. Very good yields of protoplast derived colonies were also obtained with culturing at 32°C with 0.5mg/L NAA and 0.4mg/L or 0.8 mg/L zeatin, 1 mg/L NAA and 0.8 mg/L or 1.6 mg/L zeatin, and 2 mg/L NAA and 0.4 mg/L, 0.8 mg/L or 1.6 mg/L zeatin. Good yields of protoplast-derived colonies were obtained with culturing at 32°C with 0.1 mg/L NAA and 0.4 mg/L or 0.8 mg/L zeatin, 0.5 mg/L NAA and 1.6 mg/L or 2.4 mg/L zeatin, 1 mg/L NAA and 2.4 mg/L zeatin and 2 mg/L NAA and 2.4 mg/L zeatin. Good yields were also obtained when the protoplasts were cultured at 30°C with 1 mg/L NAA and 0.4 mg/L, 0.8 mg/L, 1.6 mg/L or 2.4 mg/L zeatin. Protoplast development was similar to that seen with Coker 312. Microcalli obtained were cultured further on solid callus culture medium in accordance with the method of Example 1 and somatic embryogenic calli have been obtained for further culture.

2.4 Siokra 1-4 (*G. hirsutum*)

Protoplasts were prepared from Siokra 1-4 cotyledons following the method of Example 1, sections 1.1 - 1.3. The protoplasts were then cultured by the method of Example 1, section 1.4 except that the protoplasts were divided into two groups and cultured at 30°C or 32°C with varying concentrations of NAA (0.5, 1 or 2 mg/L) and zeatin (0.4, 0.8 or 1.6 mg/L). Very good yields of protoplast derived colonies were obtained with culturing at 32°C with 0.5 mg/L NAA and 0.4 mg/L zeatin, and 1 mg/L NAA and 0.4 mg/L or 0.8 mg/L zeatin. Protoplast development was not

observed when the protoplasts were cultured at 30°C with any combination of NAA and zeatin. Plating efficiency of Siokra 1-4 was only about half that obtained with Coker 312 protoplasts.

2.5 Sicot 189 (*G. hirsutum*)

Protoplasts were prepared from Sicot 189 cotyledons following the method of Example 1, sections 1.1 - 1.3. The protoplasts were then cultured by the method of Example 1, section 1.4 except that the protoplasts were divided into two groups and cultured at 30°C or 32°C with varying concentrations of NAA (0.5, 1 or 2 mg/L) and zeatin (0.4, 0.8 or 1.6 mg/L). Good yields of protoplast derived colonies were obtained with culturing at 32°C with 0.5 mg/L NAA and 0.4 mg/L zeatin and 1 mg/L NAA and 0.4 mg/L zeatin. Protoplast development was not observed when the protoplasts were cultured at 30°C with any combination of NAA and zeatin. The plating efficiency of Sicot 189 was less than 10% of that observed with Coker 312, but the efficiency was still sufficient to transfer some microcalli to solid medium.

EXAMPLE 3

PEG-MEDIATED TRANFORMATION OF COTTON PROTOPLASTS FOR TRANSIENT GENE EXPRESSION ANALYSIS

Cotton protoplast transformation was performed primarily as described in Negrutiu I. et al., Plant Mol. Biol. 8:363-373, 1987. Cotton protoplasts were obtained and purified as described in Example 1. Prior to transformation, an aliquot of the cotton protoplast suspension containing 500,000 protoplasts was centrifuged at 55 x g for 5 min.. The pellet was resuspended in 0.5 ml of 9% mannitol, 15 mM $MgCl_2$ $6H_2O$ and 0.1 % MES, pH 5.6 and incubated for twenty minutes at room temperature. 50 µg of naked DNA (pPG5 plasmid DNA bearing the *uidA* gene as described by Hall et al., Nature Biotechnology 14:1133-1138, 1996) was added to the protoplast suspension. Immediately, 0.5 ml of polyethylene glycol (PEG) solution (40% PEG 6000, 9% mannitol, 0.1 M $Ca(NO_3)_2$ $4H_2O$, pH 7-9) was added dropwise to give a final PEG content of 20%. The protoplast-DNA-PEG solution was then gently but thoroughly mixed by inversion. The suspension was incubated for 15 min. at room temperature with intermittent mixing every 5 min. After incubation the protoplasts were washed stepwise by adding 5 x 1 ml of 0.2M

$CaCl_2 2H_2O$ and the suspension centrifuged at 55 x g for 5 min. The second pellet was resuspended in 5-10 ml 9% mannitol supplemented with 1mM $CaCl_2 2H_2O$ and recentrifuged. The third pellet was then resuspended in 0.5 ml of 9% mannitol supplemented with 1 mM $CaCl_2 2H_2O$. The number of protoplasts remaining after transformation was determined using a haemocytometer and when necessary, the viability was determined using a standard FDA staining test (Widholm, JM, Stain Technol. 47: 189-194, 1972). The number of protoplasts recovered after transformation was usually 60% to 70% of the initial value. The protoplasts were cultured as described in Example 1.4.

Transient expression of β-glucuronidase activity was measured after 1 and 2 days in culture and was detectable in 22-24% of the protoplasts. After 5 days of culture cell division was already in progress and approximately 20% of the protoplast-derived microcolonies obtained showed β-glucuronidase activity.

EXAMPLE 4 - TRANSFORMATION OF PROTOPLASTS FOR STABLE GENE INTEGRATION

An aliquot containing 0.5 x 10^6 Coker 312 or Siokra 324 protoplasts prepared according to the method of Example 1, was resuspended in 0.5 ml 9% mannitol, 15mM $MgCl_2 6H_2O$ and 0.1% MES, pH 5.6 and incubated for 20 minutes at room temperature. 50µg of DNA (plasmid pPG5 which contains the GUS and pat genes or plasmid pB1426 which contains GUS and nptII genes) was added, followed by 0.5 ml polyethylene glycol (PEG-CMS, PEG 6000). The DNA and PEG were mixed with the protoplasts and incubated for 15 minutes. The protoplast mixture was then diluted with 1 ml $CaCl_2 H_2O$ (0.2 M), and further diluted with 1 ml $CaCl_2 H_2O$ (0.2M) four times at 5 minute intervals (for a total of 5 dilutions). The protoplasts were then rinsed with 3 ml 9M (9% mannitol and 1mM $CaCl_2 H_2O$) and resuspended in 9M. The number of protoplasts was estimated prior to embedding in alginate and culture in modified KSP medium in accordance with Example 1.

To select for stably transformed cotton cells, cultures were grown under selective conditions which favored the growth of genetically modified cells in comparison to non-modified cells. The selective agents used could be applied from day 0 onwards or preferably from day 1. When using pPG5, harboring the pat gene,

phosphinothricin (BASTA) or preferably bialophos was used. Bialophos used at concentrations of 0.1-2.0 mg/L and preferably 0.25 mg/L up to day 14, and thereafter at 1 mg/L gave the best results. When using pBI 426, harboring the nptII gene, kanamycin (25-100 mg/L) or preferably G418 (geneticin, 1-10 mg/L) was used. For the former 50 mg/L and for the latter 5 mg/L was the preferred concentration of the selection agent.

Stable transformation frequencies (percentage of initial protoplasts giving rise to an established callus) are in the 1-3 x 10^{-4} range. This is equivalent to transformation values for other species. Of the established calli obtained, 40-80% are strongly GUS positive on histochemical staining.

For Coker 312, calli were cultured further and these gave rise to somatic embryogenic areas. From these areas, somatic embryos could be isolated which showed a GUS positive response on histochemical staining indicating stable genetic integration of the foreign DNA. Some of these embryos have been shown to germinate into small plants.

CLAIMS

What is claimed is:

1. A method for regeneration of a cotton plant comprising
   (a) culturing a protoplast prepared from somatic cotton plant tissue in a culture medium, wherein said protoplast is embedded in a solid medium; and
   (b) regenerating a plant from the cultured protoplast.

2. The method of claim 1 wherein said protoplast is transformed with foreign DNA and said plant is a transgenic plant.

3. The method of claim 1 wherein said protoplast is cultured in a culture medium to form callus, foreign DNA is introduced into said callus to form transformed callus and a transgenic plant is regenerated from said transformed callus.

4. The method of claim 1, 2 or 3 wherein said culture medium further contains an antioxidant.

5. The method of claim 4 wherein said antioxidant is n-propyl gallate or glutathione.

6. The method of claim 1, 2, or 3 wherein said solid medium is alginate.

7. The method of claim 1, 2 or 3 wherein said somatic cotton plant tissue is cotyledon tissue.

8. The method of claim 1 wherein said cotton plant is Gossypium hirsutum or Gossypium barbadense.

9. A cotton plant prepared according to the method of any one of claims 1-7.

10. A cotton plant which is the progeny of a cotton plant according to claim 9.

11. A cotton seed produced by a cotton plant of claim 9 or 10.

12. A cotton plant which is derived from a cotton plant of claim 9.